University of Zurich[UZH]

Zurich Open Repository and Archive
University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2012

Inhibitor of apoptosis (IAP) proteins limit RIP3 dependent IL-1 activation

Vince, J E ; Wong, W Wei-Lynn ; Gentle, I E ; Lawlor, K E ; Allam, R ; O'Reilly, L ; Mason, K ; Gross, O ; Ma, S ; Guarda, G ; Anderton, H ; Castillo, R ; Häcker, G ; Silke, J ; Tschopp, J

Abstract: Interleukin-1b (IL-1b) is a potent inflammatory cyto- kine that is usually cleaved and activated by inflam- masome-associated caspase-1. To determine whether IL-1b activation is regulated by inhibitor of apoptosis (IAP) proteins, we treated macrophages with an IAP-antagonist ''Smac mimetic'' compound or genetically deleted the genes that encode the three IAP family members cIAP1, cIAP2, and XIAP. After Toll-like receptor priming, IAP inhibition triggered cleavage of IL-1b that was mediated not only by the NLRP3-caspase-1 inflammasome, but also by cas- pase-8 in a caspase-1-independent manner. In the absence of IAPs, rapid and full generation of active IL-1b by the NLRP3-caspase-1 inflammasome, or by caspase-8, required the kinase RIP3 and reactive oxygen species production. These results demonstrate that activation of the cell death-inducing ripop- tosome platform and RIP3 can generate bioactive IL-1b and implicate them as additional targets for the treatment of pathological IL-1-driven inflammatory responses.

DOI: https://doi.org/10.1016/j.immuni.2012.01.012

Posted at the Zurich Open Repository and Archive, University of Zurich
ZORA URL: https://doi.org/10.5167/uzh-70038
Journal Article
Published Version

Originally published at:
Vince, J E; Wong, W Wei-Lynn; Gentle, I E; Lawlor, K E; Allam, R; O'Reilly, L; Mason, K; Gross, O; Ma, S; Guarda, G; Anderton, H; Castillo, R; Häcker, G; Silke, J; Tschopp, J (2012). Inhibitor of apoptosis (IAP) proteins limit RIP3 dependent IL-1 activation. Immunity, 36(2):215-227.
DOI: https://doi.org/10.1016/j.immuni.2012.01.012

Immunity
Article

Inhibitor of Apoptosis Proteins Limit RIP3 Kinase-Dependent Interleukin-1 Activation

James E. Vince,[1,6,*] W. Wei-Lynn Wong,[2,3,5] Ian Gentle,[4,5] Kate E. Lawlor,[2,3] Ramanjaneyulu Allam,[1] Lorraine O'Reilly,[2,3] Kylie Mason,[2,3] Olaf Gross,[1] Stephen Ma,[2,3] Greta Guarda,[1] Holly Anderton,[2,3] Rosa Castillo,[1] Georg Häcker,[4] John Silke,[2,3] and Jürg Tschopp[1,7]

[1]Department of Biochemistry, University of Lausanne, 1066 Epalinges, Switzerland
[2]The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia
[3]Department of Medical Biology, the University of Melbourne, Parkville, Victoria 3010, Australia
[4]Institut für Med. Mikrobiologie und Hygiene Abt. Mikrobiologie und Hygiene Hermann-Herder-Str. 11, 79104 Freiburg, Germany
[5]These authors contributed equally to this work
[6]Present address: The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia
[7]Deceased
*Correspondence: vince@wehi.edu.au
DOI 10.1016/j.immuni.2012.01.012

SUMMARY

Interleukin-1β (IL-1β) is a potent inflammatory cytokine that is usually cleaved and activated by inflammasome-associated caspase-1. To determine whether IL-1β activation is regulated by inhibitor of apoptosis (IAP) proteins, we treated macrophages with an IAP-antagonist "Smac mimetic" compound or genetically deleted the genes that encode the three IAP family members cIAP1, cIAP2, and XIAP. After Toll-like receptor priming, IAP inhibition triggered cleavage of IL-1β that was mediated not only by the NLRP3-caspase-1 inflammasome, but also by caspase-8 in a caspase-1-independent manner. In the absence of IAPs, rapid and full generation of active IL-1β by the NLRP3-caspase-1 inflammasome, or by caspase-8, required the kinase RIP3 and reactive oxygen species production. These results demonstrate that activation of the cell death-inducing ripoptosome platform and RIP3 can generate bioactive IL-1β and implicate them as additional targets for the treatment of pathological IL-1-driven inflammatory responses.

INTRODUCTION

Release of the proinflammatory interleukin-1 (IL-1) family members IL-1α and IL-1β and their engagement of the IL-1 receptor modulates sterile inflammatory responses and activates host immune defenses against a range of human pathogens (Menu and Vince, 2011). The cleavage-induced activation and secretion of IL-1β is signaled by several cytosolic NLRs (NOD-like receptors) and the HIN-200 family member AIM2 (Schroder and Tschopp, 2010). These proteins respond to host DAMPs (damage-associated molecular patterns) and microbial PAMPs (pathogen-associated molecular patterns) to form large multimeric protein complexes known as inflammasomes. Inflammasomes recruit and activate caspase-1 either directly or indirectly via the adaptor protein ASC, leading to caspase-1 cleavage and activation of precursor IL-1β.

The most-studied inflammasome to date is NLRP3 (also known as NALP3). Mutations in NLRP3 associated with heritable cryanopanopathies cause increased inflammasome activation and these diseases are successfully treated by anti-IL-1 therapy (Menu and Vince, 2011). NLRP3 is activated by a variety of PAMPs such as bacterial pore-forming toxins and viral DNA (Hoffman and Brydges, 2011). More recently, NLRP3 inflammasome sensing of sterile DAMPs or environmental irritants that contribute to diseases such as atherosclerosis (cholesterol crystals), Alzheimer's (β-amyloid), type II diabetes (islet amyloid polypeptide, ceramide, saturated fatty acids), pulmonary fibrotic disorders (asbestos, silica dioxide), and gout (monosodium urate) has been reported (Rock et al., 2010). The diversity and number of NLRP3 stimuli suggest that NLRP3 is an important sensor for specific metabolic disturbances. Although the precise mechanism governing NLRP3 activation remains unresolved, current models propose a role for mitochondrial reactive oxygen species (ROS) generation, potassium efflux, and phagolysosomal damage (Jin and Flavell, 2010).

Until recently, IAP proteins were primarily known for inhibiting apoptotic caspase activity. In mammals, XIAP (X chromosome-linked IAP) binds and inhibits caspase-9, caspase-3, and caspase-7 through evolutionary conserved baculoviral IAP repeat (BIR) motifs and a RING finger E3 ligase domain (Gyrd-Hansen and Meier, 2010). Although they contain a similar domain structure to XIAP, the related IAP proteins cellular IAP-1 (cIAP1) and cellular IAP-2 (cIAP2) do not directly inhibit caspase proteolytic activity (Eckelman and Salvesen, 2006).

Because IAP expression can prevent cell death and may promote cancer cell survival, IAP antagonist compounds, known as Smac mimetics, have been developed. Like mature mammalian Smac, Smac mimetics bind to the BIR2 and BIR3 grooves of IAP proteins, resulting in the loss of XIAP-mediated caspase inhibition (Dynek and Vucic, 2010). An unexpected finding was that Smac mimetic targeting of cIAP1 induces cIAP1 dimerization and activation of its E3 ligase activity, resulting in ubiquitylation and proteasomal degradation of itself and cIAP2 (Darding et al., 2011; Feltham et al., 2011; Varfolomeev et al., 2007; Vince et al., 2007).

Our knowledge of IAP protein function has expanded rapidly with the development and use of Smac mimetics, and IAPs are now recognized as key regulators of mammalian immunity through their control of TNF receptor and pattern-recognition receptor (PRR) family signaling pathways. IAP inhibition can sensitize cells to TNFR1-, TLR3-, or TLR4-induced apoptosis or RIP3-dependent necroptosis by preventing cIAP1- and cIAP2-mediated ubiquitylation of core signaling components such as RIP1 kinase (Feoktistova et al., 2011; Gyrd-Hansen and Meier, 2010; He et al., 2011; Weber et al., 2010). IAPs also promote TLR2 and TLR4 cytokine production by inducing the degradation of the adaptor protein TRAF3 (Tseng et al., 2010) and control signaling from the viral RNA receptor RIG-I by catalyzing nondegradative ubiquitylation of TRAF3 and TRAF6 (Mao et al., 2010). Ubiquitylation of RIP2 by cIAP1 and cIAP2 is also required for cytokine production by the NLR family members NOD1 and NOD2, which detect bacterial peptidoglycans (Bertrand et al., 2009).

These reports and others demonstrate that IAP proteins largely promote innate immune activation by facilitating PRR-dependent transcriptional responses. Here we reveal an unexpected role for IAPs in negatively regulating inflammatory responses by limiting RIP3 kinase-dependent activation of NLRP3-caspase-1 and -caspase-8 pathways that can independently promote IL-1β precursor maturation and secretion.

RESULTS

The Smac Mimetic Compound A Induces Activation and Secretion of Caspase-1 and IL-1

To determine whether IAPs can modulate IL-1 secretion, we primed bone marrow-derived dendritic cells (BMDCs) with LPS to induce intracellular IL-1β precursor expression and then treated them with a bivalent Smac mimetic, compound A (Vince et al., 2007), and examined IL-1α and IL-1β release into the supernatant. Cells primed with LPS before compound A treatment released substantial amounts of both IL-1α and IL-1β within 6 hr, as detected by ELISA (Figures 1A and 1B).

To determine whether the IL-1β secreted in response to Smac mimetic was the mature, biologically active form and whether its release correlated with caspase-1 activation, we also analyzed cell supernatants and lysates by immunoblot (Figure 1C). LPS-primed cells treated with the NLRP3 inflammasome agonist alum promoted the secretion of processed caspase-1 and the biologically active 17 kDa IL-1β cleavage fragment (Eisenbarth et al., 2008). Similarly, compound A treatment resulted in the canonical processing and secretion of IL-1β and caspase-1 and was effective at doses as low as 100 nM (Figure 1C and Figure S1A available online). Compound A cotreatment with the NLRP3 activators alum or R837 also increased IL-1α, IL-1β, and caspase-1 activation and secretion from BMDMs compared to treatment with either agent alone (Figures 1D, 1E, S1B, and S1C). Notably, compound A-treated BMDCs that were primed with other TLR or TNF receptor ligands also caused substantial caspase-1 and IL-1β activation and secretion (Figures 1F and S1D). Despite the fact that compound A is a potent activator of noncanonical NF-κB (Vince et al., 2007), by itself it was unable to markedly induce precursor IL-1β production (Figure 1C).

The mechanism of IL-1α and IL-1β release from the cell is still unclear but may involve cell death or pore formation in the plasma membrane (Eder, 2009). To determine whether Smac mimetic increased BMDC or BMDM death or pore formation, we stained cells with propidium iodide (PI) (Figure 1G) or PI and annexin V (Figure S1E) after 8 hr of treatment, 2 hr after all IL-1 assays were performed. At this time point, LPS-primed and compound-treated cells showed no significant difference in PI or annexin V staining compared to untreated cells, whereas staurosporine, cycloheximide, and ATP all induced significant amounts of PI and annexin V uptake (Figures 1G and S1E). These data indicate that Smac mimetic-induced caspase-1 and IL-1 activation takes place before cell death occurs.

Combined Genetic Deletion of XIAP, cIAP1, and cIAP2 Recapitulates Smac Mimetic-Induced Caspase-1 and IL-1β Activation

Compound A promotes the rapid degradation of cIAP1 and cIAP2 and also antagonizes XIAP function. To investigate which IAP protein was critical in regulating IL-1β activation, we took advantage of XIAP-deficient mice and conditionally gene-targeted cIAP1 (encoded by *Birc2*) and cIAP2 (encoded by *Birc3*) mice (Gardam et al., 2011). Single, double, and triple IAP-deficient BMDMs were treated with LPS alone, or LPS followed by NLRP3 stimuli, and cell supernatants and lysates analyzed by immunoblot (Figures 2A and 2B). All IAP gene-targeted cells responded in a similar way to wild-type (WT) cells by only producing IL-1β precursor in response to LPS priming (Figure 2B). WT, single, and double IAP-deficient macrophages secreted substantial amounts of active caspase-1 and IL-1β only when treated with the NLRP3 agonists alum (Figures 2A and 2B), nigericin, or ATP (Figure S2A). In contrast, but recapitulating Smac mimetic treatment of WT macrophages, triple IAP-deficient BMDMs activated and secreted large amounts of caspase-1 and IL-1β when primed with LPS alone (Figure 2A). Consistent with the requirement for the inhibition of all three IAPs for maximal IL-1β activation, LPS-primed *Xiap*$^{-/-}$ or *Xiap*$^{-/-}$*Birc3*$^{-/-}$ BMDMs secreted large amounts of IL-1β only when treated with compound A to remove the remaining IAPs (Figure S2B). Although ELISA detection revealed that LPS priming induced some IL-1β secretion in *Xiap*$^{-/-}$*Birc3*$^{-/-}$ BMDMs, this was substantially lower than when all three IAPs were deleted or inhibited (Figures 2A and S2B). LPS treatment also induced caspase-3 and PARP cleavage in triple IAP-deficient and *Xiap*$^{-/-}$*Birc3*$^{-/-}$ cells, and to a lesser extent *Xiap*$^{-/-}$ BMDMs (Figure 2B). However, PI and Annexin V staining demonstrated that cell death in response to IAP inhibition occurs after IL-1β activation (Figures 1G and S1E and data not shown).

Both transforming growth factor-β-activated kinase 1 (TAK1) and TRAF2 have been implicated in the signaling pathways affected by Smac mimetic treatment (Darding et al., 2011; Vanlangenakker et al., 2011). TRAF2-targeted cells respond in a similar way to Smac mimetic-treated or IAP-deficient cells, with spontaneous noncanonical NF-κB activation and sensitization to death receptor killing (Grech et al., 2004; Vince et al., 2009). We therefore tested whether TRAF2- or TAK1 (encoded by *Map3k7*)-deficient BMDMs activated IL-1β similar to IAP-deficient or Smac mimetic-treated cells. However, unlike IAP-deficient cells, LysM-cre *Traf2*$^{-/-}$ and LysM-cre *Map3k7*$^{-/-}$ BMDMs

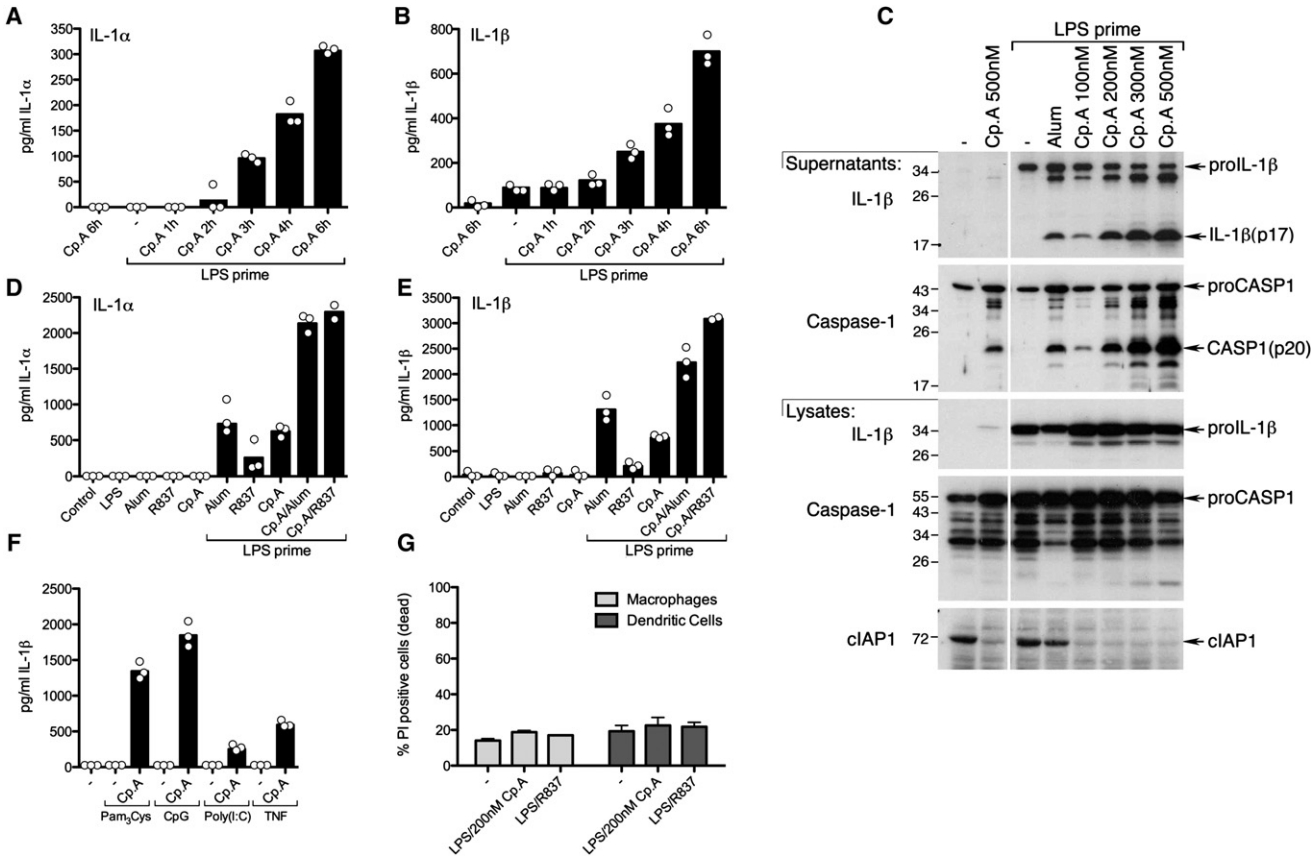

Figure 1. The Smac Mimetic Compound A Induces Caspase-1 and IL-1 Activation and Secretion from Macrophages and Dendritic Cells

(A and B) BMDCS were primed with LPS as indicated and treated with 200 nM of Cp. A for the times shown. IL-1α (A) and IL-β (B) release into the supernatant was measured by ELISA. The mean (bars) and measurements of three replicate experiments is shown.

(C) BMDMs were primed with LPS and treated with varying doses of Cp. A (100–500 nM) or alum (250 μg/ml) for 6 hr. Cell supernatants and lysates were analyzed by immunoblot.

(D and E) BMDMs were primed with LPS as indicated and then treated with alum (250 μg/ml) or R837 (10 μg/ml) ± Cp. A (200 nM) for 6 hr. IL-1α (D) and IL-β (E) release into the supernatant was measured by ELISA. The mean (bars) and measurements of three replicate experiments is shown.

(F) BMDCs were primed for 3 hr with ligands for TLR2 (P_3Cys (tripalmitoyl-S-glyceryl-Cys-Ser-4(Lys), 2 μg/ml), TLR9 (CpG (CpG-containing DNA), 2 μg/ml), TLR3 (poly(I:C) [polyinosinic:polycytidylic acid], 2.5 μg/ml), or TNFR1 (TNF, 50 ng/ml). Cells were then stimulated for 6 hr with Cp. A (200 nM) and IL-1β release was quantified by ELISA. The mean (bars) and measurements of three replicate experiments is shown.

(G) BMDMs or BMDCs were primed with LPS and then treated with 200 nM Cp. A or 10 μg/ml R837 for 8 hr. Cell viability was quantified by propidium iodide (PI) staining and flow cytometry. Error bars represent the SD of cells derived from three mice.

The results from all experiments were verified on two to three separate occasions. See also Figure S1.

did not markedly activate caspase-1 or process IL-1β in response to LPS treatment alone (Figures 2C and 2D). Furthermore, when treated with LPS plus compound A, alum, or nigericin, these cells processed and secreted IL-1β similar to WT cells (Figures 2C, 2D, S2C, and S2D). An occasional small increase in caspase-1 activation in TRAF2- or TAK1-deficient macrophages treated with compound A alone was not significant and did result in increased IL-1β secretion (Figures 2C, S2C, and S2D).

Inhibition of IAP Proteins Results in NLRP3-Caspase-1 Inflammasome-Dependent and -Independent IL-1 Activation

Our results suggested that the three IAPs act together to suppress caspase-1 activation. We therefore examined which inflammasome might be activated by loss of IAP function. Deletion of NLRP3 reduced compound A-stimulated IL-1α and IL-1β secretion to a similar extent as observed in *Pycard*$^{-/-}$ (also known as *Asc*) or *Casp1*$^{-/-}$ BMDCs (Figures 3A, 3B, and S3B) and BMDMs (Figures 3C and S3A). Consistent with compound A causing activation of NLRP3, caspase-1 processing and its secretion was abolished in *Nlrp3*$^{-/-}$ and *Pycard*$^{-/-}$ BMDMs but unaffected in NLRC4 inflammasome-deficient BMDMs (Figures 3B, 3C, S3A, and S3C).

The failure of caspase-1 to activate in *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, or *Casp1*$^{-/-}$ (which also lack caspase-11 [Kayagaki et al., 2011]) cells did not completely prevent compound A-induced secretion of IL-1β (Figures 3A–3C and S3A) or IL-1α (Figure S3B). It has been described that TWEAK ligand can mimic compound A by reducing the amounts of cIAP1, cIAP2, and TRAF2 (Varfolomeev et al., 2007; Vince et al., 2008). However, TWEAK treatment did

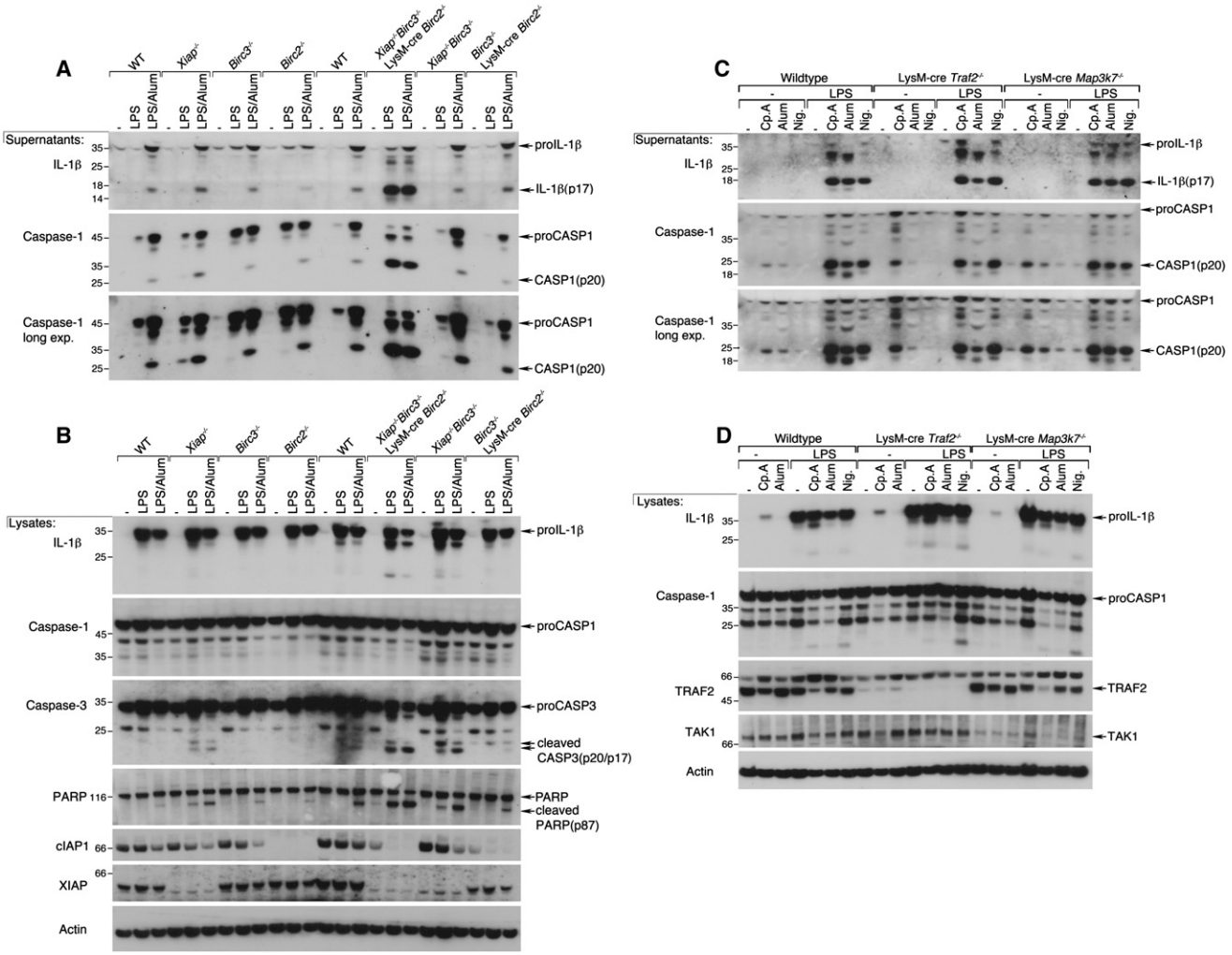

Figure 2. Combined Genetic Deletion of XIAP, cIAP1, and cIAP2 Recapitulates Smac Mimetic-Induced Caspase-1 and IL-1β Activation

(A and B) BMDMs of the indicated genotype were primed with LPS for 3 hr as indicated and then stimulated with alum (200 μg/ml) for a further 5 hr. Cell supernatants (A) or lysates (B) were analyzed by immunoblot.

(C and D) BMDMs of the indicated genotype were primed with LPS for 3 hr as indicated and then stimulated for 6 hr with Cp. A (200 nM) or alum (250 μg/ml) or for 1 hr with nigericin (4 μM). Cell supernatants (C) and lysates (D) were analyzed by immunoblot.

The results represent data obtained from two to six mice of each genotype. See also Figure S2.

not activate caspase-1 or IL-1β substantially (Figure S3A). This further supports the idea that full activation of caspase-1 and IL-1β requires inhibition of XIAP as well as cIAP1 and cIAP2.

Collectively, these data show that Smac mimetics must target XIAP, cIAP1, and cIAP2 for maximal activation of NLRP3-caspase-1. However, the absence of NLRP3, ASC (encoded by *Pycard*), or caspase-1 fails to completely prevent Smac mimetic-induced IL-1β maturation, suggesting that IAP proteins also regulate an alternate IL-1β activating pathway that is inflammasome independent.

RIP3 Is Required for Efficient Smac Mimetic-Induced IL-1 Activity

We considered whether RIP1 or RIP3 played a role in Smac mimetic-induced IL-1 activation because IAP depletion can promote the formation of RIP1 and RIP3 signaling complexes (He et al., 2009). The deletion of RIP3 decreased Smac mimetic-induced activation and secretion of caspase-1 and IL-1 in BMDCs and BMDMs after LPS-TLR4 (Figures 3A, 3D, S3B, and S3D), Pam_3Cys-TLR2, or CpG-TLR9 priming (Figure 3E). Because Smac mimetic-induced IL-1 secretion was lower in *Ripk3*$^{-/-}$ BMDCs when compared in parallel to *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, and *Casp1*$^{-/-}$ BMDCs (Figures 3A, 3E, and S3B), this suggests that RIP3 is required for optimal NLRP3-caspase-1 inflammasome-dependent and -independent IL-1 activation. Experiments with increased cell numbers revealed that, although markedly reduced, some processing of caspase-1 and IL-1β could still occur in RIP3-deficient BMDMs (Figures S3D and S4G). Nevertheless, these results indicate that IAPs suppress RIP3 signaling and that RIP3 is a regulator of the NLRP3

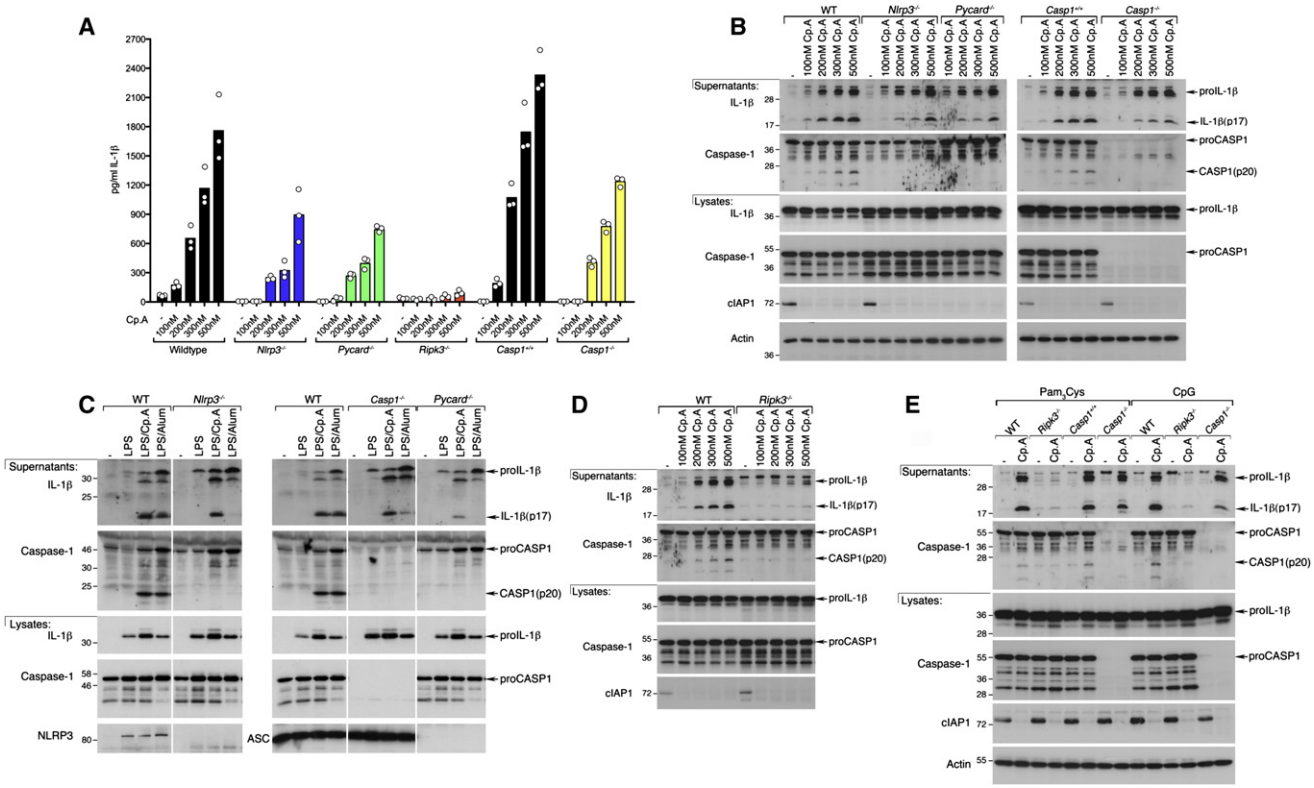

Figure 3. IAP Inhibition Activates IL-1 by NLRP3-Caspase-1 Inflammasome-Dependent and -Independent Mechanisms that Both Require RIP3

(A and B) BMDCs of the indicated genotype were primed with LPS and then stimulated for a further 6 hr with the indicated doses of Cp. A. IL-1β levels in the supernatants were detected by ELISA (A) and cell supernatants and lysates also analyzed by immunoblot (B). The mean (bars) and measurements of three replicate experiments is shown (A).

(C) BMDMs of the indicated genotype were treated with Cp. A (200 nM) or alum (250 μg/ml) for 6 hr after LPS priming. Cell supernatants and lysates were analyzed by immunoblot.

(D) WT or *Ripk3*$^{-/-}$ BMDCs were primed with LPS and then stimulated with the indicated doses of Cp. A for a further 6 hr. Cell supernatants and lysates were analyzed by immunoblot.

(E) BMDCs of the indicated genotype were primed with Pam_3Cys (2 μg/ml) or CpG (2 μg/ml) for 3 hr and then stimulated with 200 nM Cp. A for a further 6 hr. Cell supernatants and lysates were analyzed by immunoblot.

All data were confirmed on two to four separate occasions. See also Figure S3.

inflammasome and IL-1 activation. In contrast, loss of RIP3 had no impact on alum-, R837-, ATP-, or nigericin-induced NLRP3 activity (Figure S3D and not data not shown), demonstrating that RIP3 is not required for optimal NLRP3 activation by other stimuli.

Because RIP3 activation usually requires RIP1 kinase function (Cho et al., 2009; He et al., 2009; Zhang et al., 2009), we tested whether the RIP1 kinase inhibitor Necrostatin-1 (Degterev et al., 2008) affected Smac mimetic-induced IL-1β activation. However, treatment of LPS-primed BMDMs with Necrostatin-1 did not alter compound A-induced IL-1β secretion (Figure 4A). Similarly, although lysosome function and protease release or activation has been implicated in NLRP3-dependent IL-1β activation (Hornung and Latz, 2010), inhibition of lysosome function, cathepsins, and calpains with Bafilomycin A1, NH_4Cl, CA-074-Me, or E64d, respectively, did not alter Smac mimetic-induced IL-1β maturation in LPS-primed WT, *Nlrp3*$^{-/-}$, or *Pycard*$^{-/-}$ BMDMs (Figures 4A and 4B).

RIP3-Dependent Reactive Oxygen Species Production Is Required for Efficient Smac Mimetic-Induced IL-1β Activation

RIP3 signaling can promote mitochondrial ROS production (Cho et al., 2009; Vanlangenakker et al., 2011; Zhang et al., 2009), and it has also been proposed that mitochondrial damage and ROS generation can facilitate NLRP3-dependent IL-1β activation (Nakahira et al., 2011; Zhou et al., 2011). However, antioxidants commonly used to inhibit ROS accumulation can impair LPS induction of IL-1β precursor and NLRP3 (Bauernfeind et al., 2011; van de Veerdonk et al., 2010) and is consistent with a role for ROS in LPS transcriptional responses (Figures S4A–S4D; Gloire et al., 2006).

To circumvent the effects antioxidant treatment may have on LPS priming, we treated cells with antioxidants after LPS priming. We observed that four different antioxidants added after LPS priming markedly inhibited the activation of caspase-1 and IL-1β in response to compound A (Figure 4C). Antioxidant

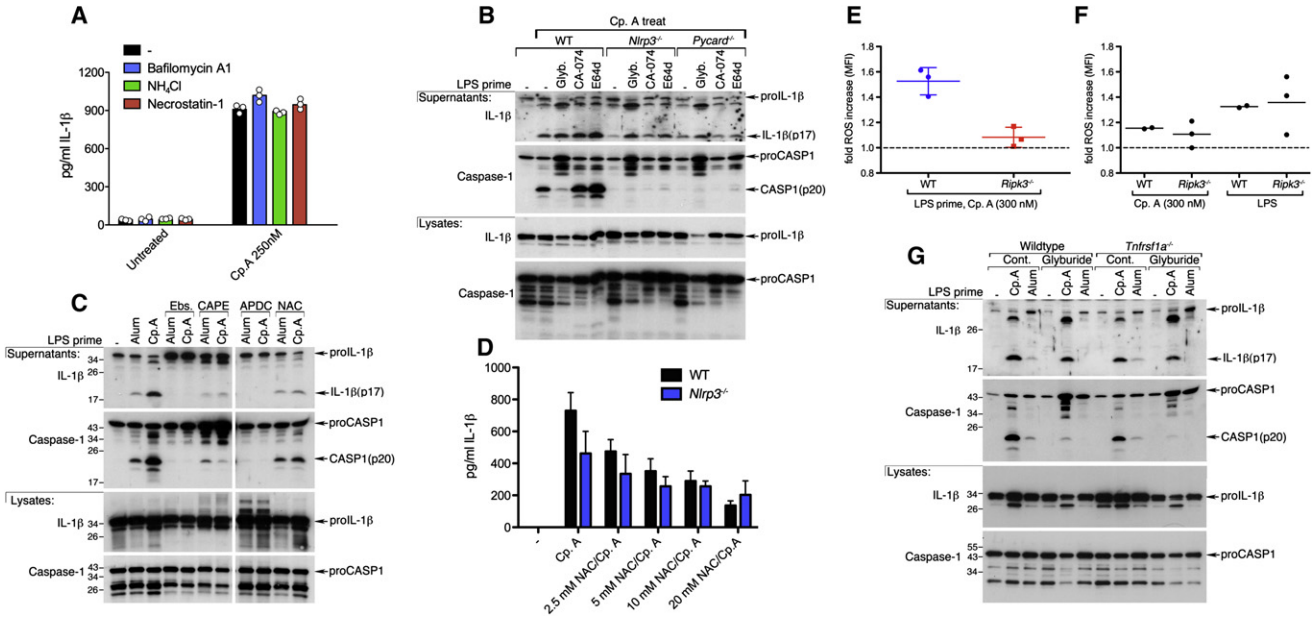

Figure 4. RIP3-Induced ROS Is Required for Efficient Smac Mimetic-Mediated IL-1β

(A) RIP1 kinase activity or lysosomal function do not contribute to Cp. A-induced IL-1β activation. BMDMs were primed with LPS and in the last 20 min of priming, cells were treated with NH_4Cl (20 mM), Bafilomycin A1 (150 nM), or Necrostatin-1 (50 μM) and then incubated with Cp. A for a further 6 hr. IL-1β release was measured by ELISA. The mean (bars) and measurements of three replicate experiments is shown.

(B) BMDMs of the indicated genotype were primed with LPS. In the last 20 min of priming, cells were treated with Glyburide (200 μM), CA-074-Me (20 μM), or E64d (20 μM) and then incubated with Cp. A (200 nM) for a further 6 hr. Cell supernatants and lysates were analyzed by immunoblot.

(C) BMDMs were primed with LPS and in the last 20 min of priming were incubated with ROS scavenging compounds: Ebselen (210 μM), CAPE (Caffeic acid phenethyl ester, 210 μM), APDC ((2*R*,4*R*)-4-aminopyrrolidine-2,4-dicarboxylate, 100 μM), or NAC (N-Acetyl-L-Cysteine, 20 mM). Cells were then stimulated for a further 6 hr with alum (200 μg/ml) or Cp. A (200 nM) and cell supernatants and lysates were analyzed by immunoblot.

(D) BMDMs were primed with LPS (3 hr) then treated with NAC for 15 min prior to Cp. A (300 nM) addition for a further 6 hr. IL-1 release was measured by ELISA. The error bars show the SD of measurements obtained from three mice of each genotype.

(E and F) WT and *Ripk3*$^{-/-}$ BMDMs were treated with 5 μM of CellROX deep red prior to LPS priming (2.5 hr) and Cp. A (300 nM) stimulation for 5 hr. Alternatively cells were treated with LPS (7.5 hr) and Cp. A (5 hr) alone. The fold change in mean fluorescence intensity (MFI) for LPS-primed and Cp. A-treated cells over cells treated with LPS alone (E) or for LPS or Cp. A treatment alone over nonstimulated cells (F) is depicted. Propidium iodide-positive cells (less then 20% of cells for both genotypes and all treatments) were excluded from the analysis. The data was collected from cells derived from three mice of each genotype (symbols) with the mean and SD (E) presented.

(G) WT and *Tnfrsf1a*$^{-/-}$ BMDMs were primed with LPS and then treated ±glyburide (200 μM) for a further 30 min as indicated. Cells were then stimulated with Cp. A (200 nM) or alum (250 μg/ml) for 6 hr and cell supernatants and lysates were analyzed by immunoblot. The results obtained in (A) were verified on two (NH_4Cl and Bafilomycin A1) or four (Necrostatin-1) occasions.

The data obtained in (C), (E), (F), and (G) were verified on two to three separate occasions. See also Figure S4.

treatment also inhibited compound A-induced NLRP3-caspase-1-independent secretion of IL-1β (Figure 4D). Propidium iodide staining and LDH release assays indicated that compound A and the antioxidant N-acetyl cysteine (NAC) were no more toxic than other NLRP3 stimuli such as alum, yet at the same time NAC prevented LPS-primed and compound A-treated BMDMs from secreting IL-1β (Figures S4E–S4G).

To determine whether RIP3 activation might induce ROS after IAP inhibition, we compared ROS production in WT and RIP3-deficient BMDMs. Whereas WT macrophages showed a robust increase in ROS amounts after LPS priming and compound A treatment, ROS generation in *Ripk3*$^{-/-}$ BMDMs was impaired (Figure 4E). In contrast, LPS or compound A stimulation alone induced less ROS, which was similar between WT and *Ripk3* $^{-/-}$ BMDMs (Figure 4F). Consistent with NAC inhibition of ROS, and ROS being an effecter of RIP3-dependent killing, *Ripk3*$^{-/-}$ BMDMs, or WT BMDMs treated with NAC, were partially protected from cell death induced by prolonged (19 hr) treatment with LPS and compound A (Figure S4H).

Compound A treatment has been shown to induce TNF production in a subset of tumor cell lines (Vince et al., 2007). However, TNF-TNFR1 signaling was not involved in compound A-induced IL-1β maturation because *Tnfrsf1a*$^{-/-}$ BMDMs secreted similar amounts of active caspase-1 and IL-1β when compared to WT BMDMs (Figure 4G). To further distinguish between compound A-induced NLRP3-dependent and -independent IL-1β activation, we treated cells with glyburide, an inhibitor of NLRP3 activity (Lamkanfi et al., 2009). Glyburide prevented caspase-1 activity in both WT and *Tnfrsf1a*$^{-/-}$ macrophages primed with LPS and treated with compound A but, unlike alum treatment, glyburide did not completely prevent compound A-induced IL-1β processing and secretion in either WT, TNFR1-, NLRP3-, or ASC-deficient cells (Figures 4B and 4G).

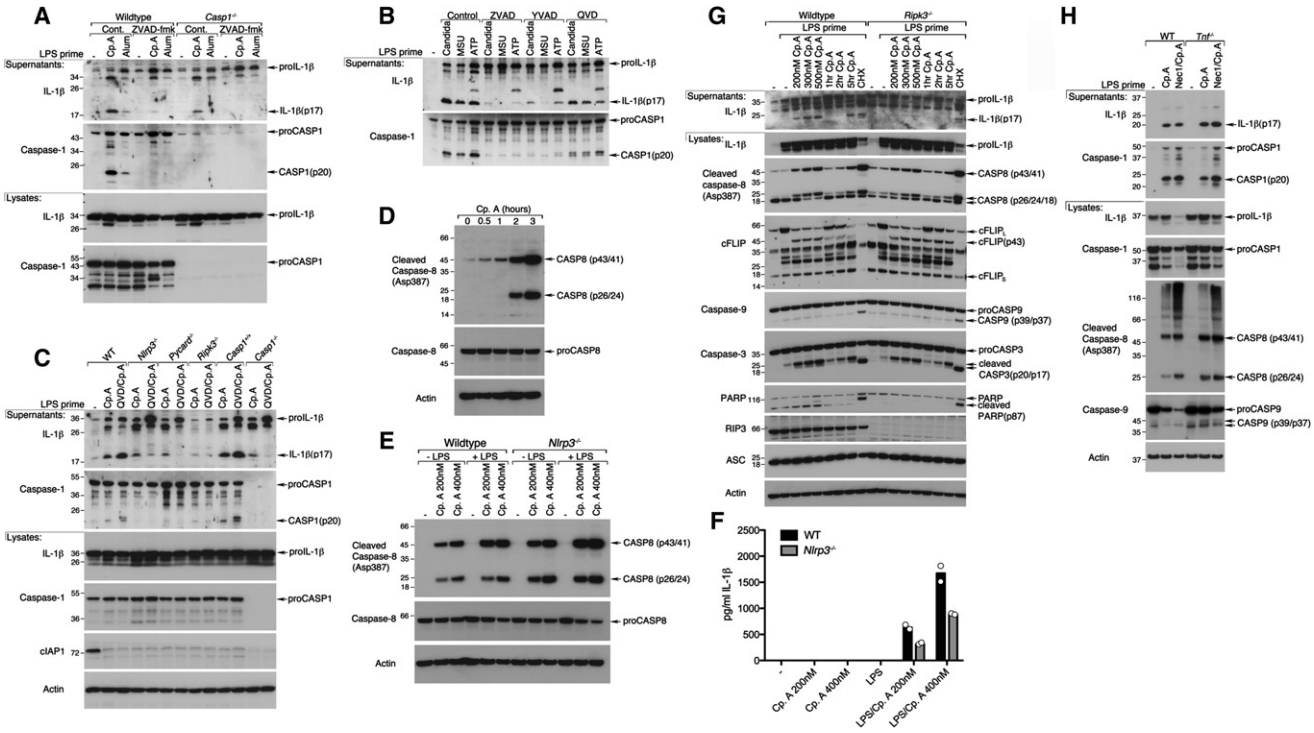

Figure 5. Pan-caspase Inhibition Prevents Caspase-1-Independent IL-1β Activation Caused by Smac Mimetic Treatment

(A) BMDMs of the indicated genotype were primed with LPS. In the last 20 min of priming, cells were incubated with 50 μM of Z-VAD-fmk. Cells were then stimulated for a further 6 hr with Cp. A (200 nM) or alum (200 μg/ml) and cell supernatants and lysates were analyzed by immunoblot.

(B) BMDMs were primed with LPS. In the last 20 min of priming, cells were incubated with 20 μM of the pan-caspase inhibitors Q-VD-Oph (QVD), Z-VAD-fmk (ZVAD), or the caspase-1 inhibitor Y-VAD-fmk (YVAD) before treatment with the indicated NLRP3 agonists for a further 6 hr. Cell supernatants were analyzed by immunoblot.

(C) BMDCs of the indicated genotype were primed with LPS. In the last 20 min of priming, cells were incubated with 10 μM of Q-VD-Oph and then stimulated for a further 6 hr with Cp. A (200 nM). Cell supernatants and lysates were analyzed by immunoblot.

(D) BMDMs were stimulated with Cp. A (300 nM) as indicated and cell lysates analyzed by immunoblot.

(E and F) WT and *Nlrp3*$^{-/-}$ BMDMs were treated as indicated with Cp. A for 6 hr ± LPS priming. Cell lysates were analyzed by immunoblot (E) and IL-1β secretion into the supernatant was measured by ELISA (F). The mean (bars) and measurements of two mice of each genotype are shown (F).

(G and H) LPS-primed BMDCs from WT or *Ripk3*$^{-/-}$ mice were treated as indicated with Cp. A or cycloheximide (CHX, 20 μg/ml) (G). Alternatively, LPS-primed BMDMs from WT or *Tnf*$^{-/-}$ mice were treated ±Necrostatin-1 (50 μM) for 20 min before incubation with Cp. A (200 nM) for a further 6 hr (H). Cell supernatants and lysates for (G) and (H) were analyzed by immunoblot.

The data presented in (A) and (C) was repeated on two separate occasions and the data presented in (G) and (H) verified on two to four separate occasions. See also Figures S4 and S5.

Collectively, these data argue that in LPS-primed cells, IAP inhibition causes RIP3-dependent ROS production and this promotes IL-1β maturation and secretion by NLRP3-caspase-1 inflammasome-dependent and -independent mechanisms. These pathways do not involve autocrine TNF production or lysosomal destabilization, both of which have previously been implicated in IAP or NLRP3 signaling in other circumstances.

Caspase Activity Is Required for Smac Mimetic-Induced NLRP3-Caspase-1-Independent IL-1β Maturation

To determine whether caspase inhibition could prevent caspase-1-independent Smac mimetic-induced IL-1β maturation, we treated cells with the pan-caspase inhibitors Z-VAD-fmk and Q-VD-Oph. BMDMs treated with Z-VAD-fmk prior to compound A incubation blocked both NLRP3-caspase-1-dependent and -independent IL-1β activation (Figure 5A). In contrast, Q-VD-Oph was less effective at inhibiting IL-1β maturation in response to well-characterized NLRP3 agonists (Figure 5B) and, surprisingly, markedly enhanced compound A-mediated IL-1β secretion (Figures 5C and S4I).

Caspase-8 inhibits RIP3 signaling, that if left unchecked causes embryonic lethality (Kaiser et al., 2011; Oberst et al., 2011). We reasoned that because Q-VD-Oph does not completely block caspase-1 activity (Figure 5B), but does effectively inhibit caspase-8, it may allow increased RIP3 signaling upon compound A stimulation, leading to enhanced NLRP3-caspase-1 cleavage of IL-1β. Consistent with this hypothesis, when we prevented compound A-induced NLRP3-caspase-1 activation by using *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, or *Casp1*$^{-/-}$ BMDCs, the effect of Q-VD-Oph was reversed, and it reduced compound A-induced IL-1β activation in these cells (Figures 5C and S4I).

If another caspase can process IL-1β independently of caspase-1, other caspase-activating signals should be able to promote IL-1β maturation in a caspase-1-independent manner.

Treatment of LPS-primed BMDMs with staurosporine, cycloheximide, or UVB radiation resulted in both caspase-1-dependent and -independent processing of IL-1β that occurred independent of RIP3 and was therefore inhibited by Z-VAD-fmk and Q-VD-OPh (Figures S5A–S5D). However, as with Smac mimetic treatment, cycloheximide, staurosporine and UVB irradiation triggered IL-1β activation and secretion was blocked by antioxidants (Figure S5E) and occurred independent of ATP release and activation of the purinergic P2X7 receptor (Figure S5F).

Caspase-8 Mediates NLRP3-Caspase-1-Independent IL-1β Maturation

We examined caspase processing in Smac mimetic-treated cells to determine which caspase other than caspase-1 might be cleaving IL-1β. We observed substantial caspase-8 processing and $cFLIP_L$ depletion in compound A-treated WT, *Nlrp3*$^{-/-}$, *Ripk3*$^{-/-}$, and *Tnf*$^{-/-}$ BMDMs or BMDCs (Figures 5D, 5E, 5G, and 5H), and this correlated with NLRP3-caspase-1-independent IL-1β secretion (Figure 5F). Unlike cycloheximide treatment, compound A did not induce substantial caspase-9 cleavage, caspase-3 processing to the mature p17 subunit, or PARP cleavage in this time frame (Figure 5G). Notably, compound A-induced caspase-8 processing and IL-1β secretion was not blocked by Necrostatin-1 in LPS-primed WT and *Tnf*$^{-/-}$ BMDMs (Figure 5H).

Consistent with our data obtained with primary macrophages, RAW 264.7 (which lack ASC) (Pelegrin et al., 2008) and C57BL/6 v-myc/v-raf ($WT^{v\text{-}myc/v\text{-}raf}$) immortalized (Bauernfeind et al., 2009) macrophages secreted mature IL-1β in response to LPS priming and Smac mimetic or cycloheximide treatment, and this occurred in the absence of caspase-1 processing (Figures 6A, S6A, and S6B). Because ASC expression in $WT^{v\text{-}myc/v\text{-}raf}$ cells is minimal when compared to BMDCs (Figure 6B), this suggests that inflammasome activation in these cells is inefficient. Notably, release of mature IL-1β into the supernatant in both RAW 264.7 and $WT^{v\text{-}myc/v\text{-}raf}$ macrophages correlated with caspase-8, but not caspase-9, processing (Figures 6A and S6B).

These results led us to hypothesize that caspase-8 is capable of processing precursor IL-1β. Although it was less efficient when compared to caspase-1, recombinant caspase-8 cleaved purified precursor IL-1β into the same mature 17 kDa fragment (Figure S6C). In contrast, caspase-9 was markedly less efficient at cleaving IL-1β than caspase-8. Because caspase-8 deletion is embryonic lethal (Varfolomeev et al., 1998) and is also required for myelomonocytic progenitor differentiation (Kang et al., 2004), we next knocked down caspase-8 in $WT^{v\text{-}myc/v\text{-}raf}$ and RAW 264.7 macrophages by shRNA targeting. Depletion of caspase-8 consistently decreased LPS- and compound A-mediated IL-1β maturation for four different caspase-8 shRNAs (Figures 6C, S6D, and S6E). A fifth caspase-8 shRNA (no. 81) resulted in poor growing cells that constitutively secreted IL-1β (Figure 6C).

Although these data imply that caspase-8 can activate IL-1β independently of caspase-1, they do not exclude the possibility that another caspase activated downstream of caspase-8 is responsible for caspase-1-independent IL-1β maturation. To test this, we generated BMDMs and BMDCs from caspase-3-, caspase-7-, caspase-2-, or caspase-11-deficient mice. Because caspase-8 can cleave Bid to induce Bax-Bak activation, we also examined BMDMs derived from *Bid*$^{-/-}$ mice or lethally irradiated mice that had been reconstituted with *Bax*$^{-/-}$*Bak1*$^{-/-}$ fetal liver. However, activation of IL-1β in all these cells was similar to WT cells when they were primed with LPS and treated with compound A, alum, or ATP (Figures 6D, 6E, and S7A–S7E).

Collectively, these results argue that as a result of Smac mimetic-mediated IAP inhibition, caspase-8, but not downstream effector caspases, can cleave precursor IL-1β into its mature biologically active 17 kDa fragment, and that this represents an alternate mechanism for IL-β activation independent of inflammasome activity.

IAP Depletion Induces Ripoptosome Formation and Promotes RIP3-Dependent Caspase-8 Modification and Activity

IAP depletion has recently been shown to induce the spontaneous complexing of RIP1 with caspase-8 and FADD (Feoktistova et al., 2011; Tenev et al., 2011). This complex, termed the ripoptosome, can activate both RIP3 and caspase-8 signaling. We hypothesized that IAP depletion in macrophages is likely to induce ripoptosome formation which drives IL-1β activation because, consistent with ripoptosome activity, compound A-induced IL-1β maturation and secretion (1) occurs independent of TNF or TNFR1, (2) does not involve Bax and Bak, (3) is maximal when all three IAPs are deleted, (4) requires RIP3 signaling, and (5) depends partially on caspase-8. In agreement with these observations and ripoptosome assembly, BMDMs treated with compound A alone was sufficient to cause increased RIP1 associated with immunoprecipitated caspase-8, and this association was increased if cells were primed with LPS before compound A treatment (Figure 7A). Therefore, IAPs appear to suppress ripoptosome formation that when activated can drive the generation of bioactive IL-1β.

Because *Ripk3*$^{-/-}$ BMDCs secrete less IL-1β upon TLR priming and compound A stimulation when compared in parallel with *Nlrp3*$^{-/-}$, *Pycard*$^{-/-}$, or *Casp1*$^{-/-}$ cells (Figures 3A and 3E), this suggests that RIP3 deficiency must also impair caspase-8-dependent IL-1β maturation. Although compound A-induced caspase-8 processing is not substantially decreased in LPS-primed *Ripk3*$^{-/-}$ cells (Figures 5G and 7B), cleaved caspase-8 modification, presumably ubiquitylation, was markedly reduced when RIP3 was deleted (Figure 7B).

Caspase-8 ubiquitylation is required for caspase-8 sequestering into lipid raft membranes and full caspase-8 activity (Jin et al., 2009). Consistent with this, we observed that in response to LPS and compound A treatment, cleaved and modified caspase-8 fractionates preferentially into detergent-resistant membranes (Figure 7B). Importantly, decreased caspase-8 modification in *Ripk3*$^{-/-}$ BMDMs correlated with decreased compound A-induced caspase-8 activity when compared to WT BMDMs (Figure 7C). Compound A-induced caspase-8 activity was also efficiently inhibited by the antioxidant NAC (data not shown), implicating RIP3-generated ROS in both NLRP3-caspase-1- and caspase-8-mediated IL-1β maturation (Figure 4D).

DISCUSSION

In this study, we demonstrate that IAP proteins inhibit ripoptosome formation and RIP3 signaling to prevent NLRP3-caspase-1- and caspase-8-dependent activation of IL-1.

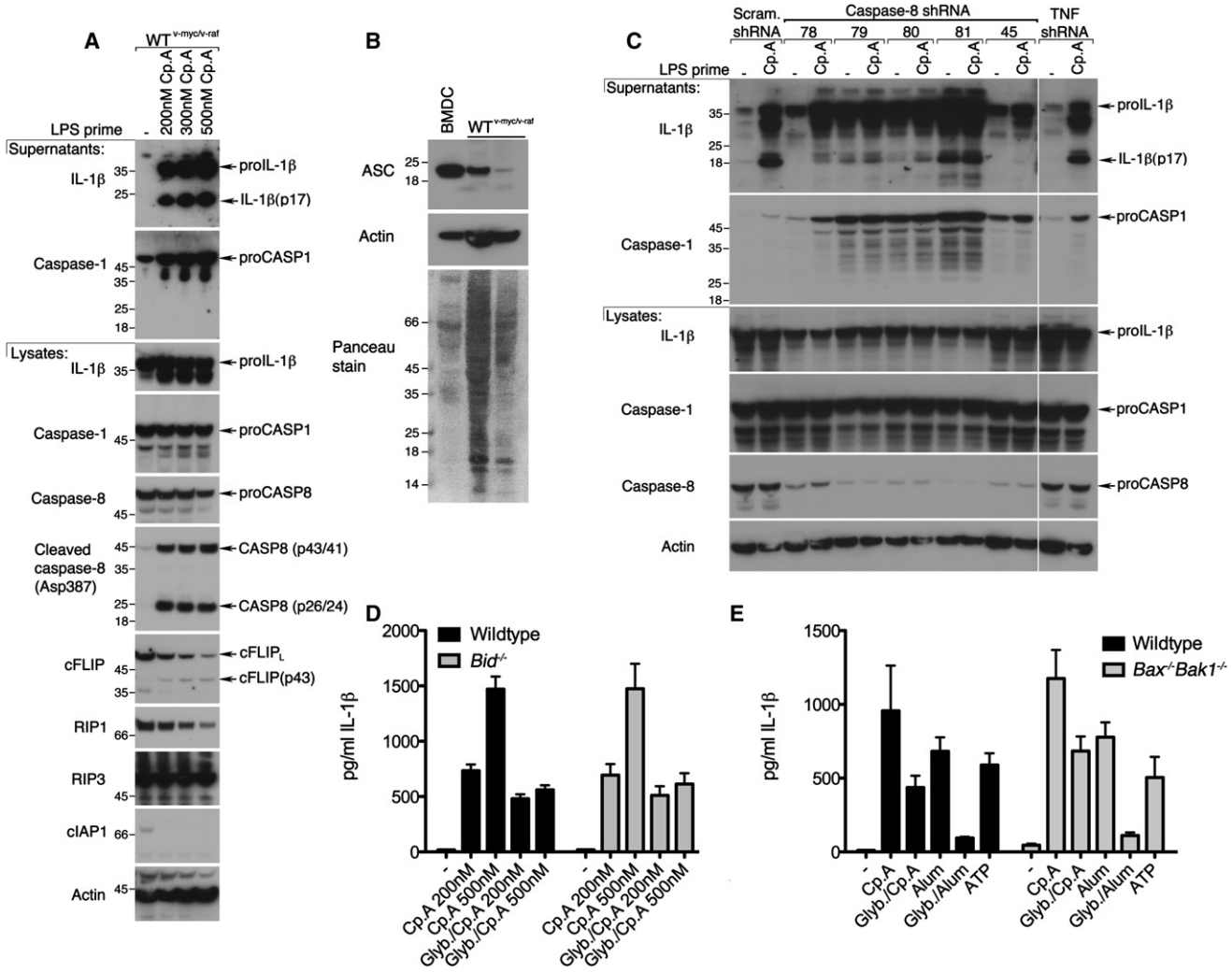

Figure 6. IAP Inhibition Causes Caspase-8 Cleavage of Precursor IL-1β that Is Independent of NLRP3-Caspase-1

(A) $WT^{v\text{-}myc/v\text{-}raf}$ macrophages were primed with LPS and treated with the indicated doses of Cp. A for 6 hr. Cell supernatants and lysates were analyzed by immunoblot.

(B) ASC levels in BMDCs were compared to $WT^{v\text{-}myc/v\text{-}raf}$ macrophages by immunoblot. Actin and Panceau staining indicate protein loading.

(C) $WT^{v\text{-}myc/v\text{-}raf}$ macrophages expressing five different shRNAs targeting caspase-8, or scrambled and TNF control shRNAs, were primed with LPS followed by Cp. A (300 nM) treatment for 6 hr. Cell supernatants and lysates were analyzed by immunoblot.

(D and E) $Bid^{-/-}$ (D) or $Bax^{-/-}Bak1^{-/-}$ (E) BMDMs were primed with LPS ± glyburide (200 μM) treatment in the last 20 min of priming. Cells were subsequently stimulated with Cp. A or alum (250 μg/ml) for 6 hr or ATP (5 mM) for 1 hr. IL-1β secretion into the supernatant was measured by ELISA. Error bars represent the SD of nine replicates from three mice of each genotype.

Data in (A)–(C) were verified on three separate occasions and data in (E) on two separate occasions. See also Figures S6 and S7.

RIP3-mediated IL-1 secretion precedes cell death, and this suggests that RIP3 may influence inflammatory responses independent of its ability to induce necroptosis (Wallach et al., 2011). The identification of these pathways leading to IL-1 activation, and their suppression by IAPs, may have important implications for RIP1-RIP3-dependent signaling that has been associated with the control of viral infection or pathologies such as pancreatitis (Vandenabeele et al., 2010).

IAP depletion triggers NLRP3-caspase-1 inflammasome-dependent IL-1 activation and also enhances IL-1 secretion induced by other NLRP3 agonists. Removal of the three IAP proteins XIAP, cIAP1, and cIAP2 is required for maximal NLRP3-caspase-1 and IL-1β activation. Therefore it is likely that under normal conditions, or in response to immune cell recognition of specific PAMPs or DAMPs, these three IAP proteins act in concert to suppress inappropriate or excess IL-1 activity.

Although caspase-1 activation is regarded as the primary mechanism for producing bioactive IL-1β, FasL can induce IL-1β maturation and secretion that is independent of caspase-1 but is blocked by pan-caspase inhibition (Miwa et al., 1998). Our data demonstrate that IAP depletion, and a number of other caspase-activating agents, can also induce IL-1β maturation in the absence of caspase-1 activity, and that this is most probably mediated through IL-1β precursor cleavage by caspase-8. By extension,

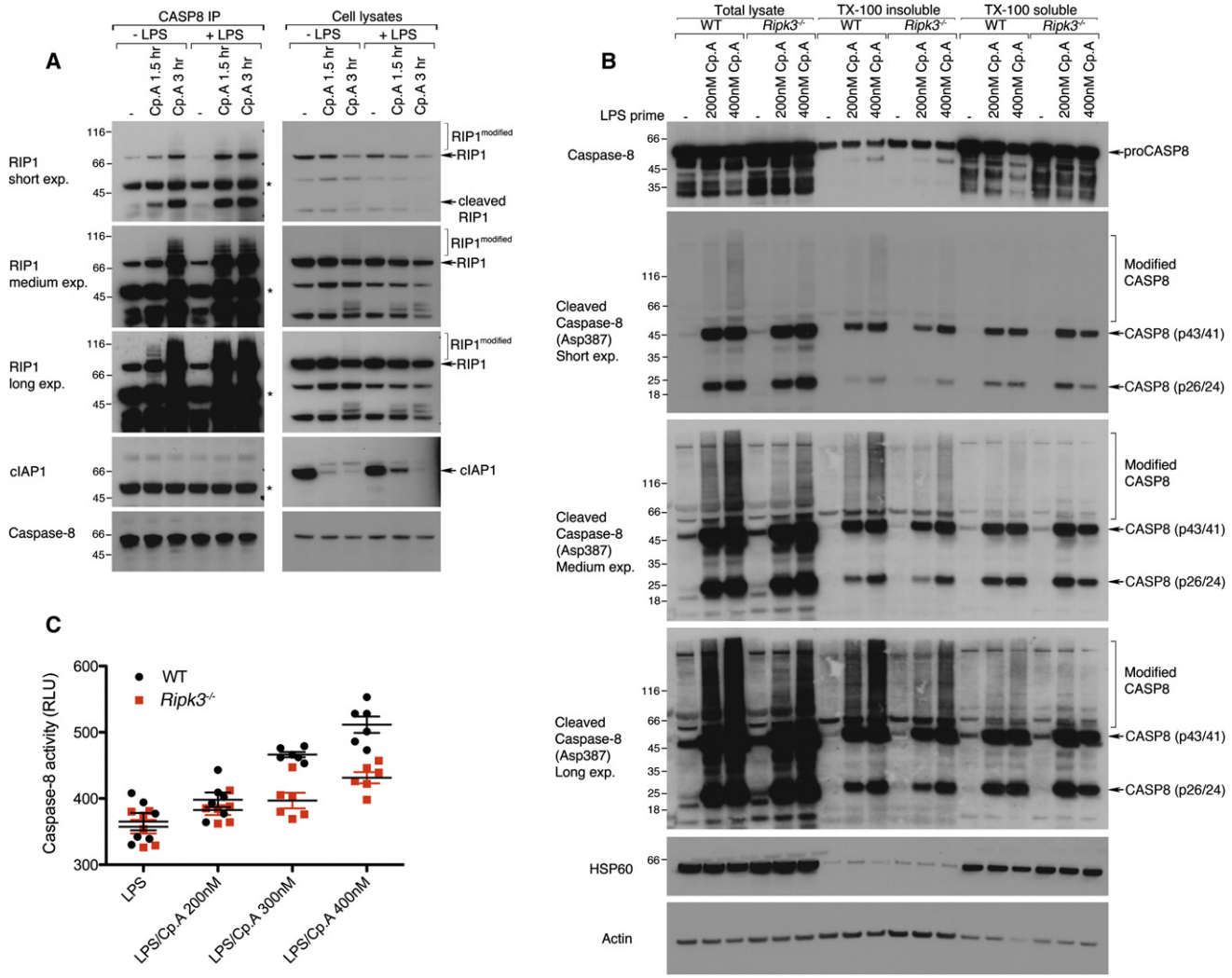

Figure 7. IAP Depletion Induces Ripoptosome Formation and Promotes RIP3-Dependent Caspase-8 Modification and Activity

(A) BMDMs were primed with LPS (2 hr) and then treated with Q-VD-Oph (10 μM) and Cp. A as indicated. Caspase-8 was immunoprecipitated from cell lysates and RIP1 association analyzed by immunoblot. Asterisk indicates antibody IgG heavy chain cross reactivity.

(B) WT and *Ripk3*$^{-/-}$ BMDMs were primed with LPS and then treated with Cp. A (4 hr) as indicated. Cells were lysed directly in reducing sample buffer (total lysate) or fractionated into Triton X-100 insoluble and soluble fractions and analyzed by immunoblot.

(C) WT and *Ripk3*$^{-/-}$ BMDMs were primed with LPS for 2 hr and then treated for a further 4 hr with Cp. A as indicated. Cellular caspase-8 activity was then measured (Promega, Caspase-8 Glo assay kit). Measurements were performed in duplicate for three mice of each genotype (symbols). Error bars represent the SD.

The data in (A) were verified on two separate occasions and the data in (B) and (C) verified on three separate occasions.

these data suggest that Fas activation of caspase-8 would also promote IL-1β activity. Caspases activated downstream of caspase-8 are unlikely to contribute to IL-1β activation because caspase-2-, caspase-3-, caspase-7-, and caspase-11-deficient cells show normal IL-1β activation in response to IAP inhibition. This is consistent with inefficient IL-1β precursor cleavage by recombinant caspase-3, caspase-7, and caspase-9 and a previous study suggesting that caspase-8 is required for TLR3- or TLR4-induced IL-1β secretion (Lüthi et al., 2009; Maelfait et al., 2008).

IAP depletion in macrophages induces ripoptosome formation, which can promote RIP3 and caspase-8 signaling and is therefore likely to drive subsequent IL-1β activation. Because RIP1 kinase activity promotes ripoptosome assembly and is usually required for RIP3 signaling, it was therefore surprising that the RIP1 kinase inhibitor Necrostatin-1 failed to prevent Smac mimetic-mediated IL-1β maturation. It is possible that LPS priming (which contributes to ripoptosome activation) induces RIP1 kinase activity, and that this pool of "preactivated" RIP1 is not inhibited by subsequent Necrostatin-1 and Smac mimetic treatment. Alternatively, upon IAP depletion, RIP3 may be activated independent of RIP1, as suggested for viral-induced RIP3 signaling (Upton et al., 2010). The unexpected role for RIP3 in promoting caspase-8 modification and activity also implies that RIP3 facilitates a negative feedback loop, because active caspase-8 can process and possibly inactivate RIP1, RIP3, or CYLD (Feng et al., 2007; Lin et al., 1999; O'Donnell

et al., 2011), which is likely to dampen ripoptosome function. How RIP3 promotes caspase-8 modification remains undetermined, but in this context it is noteworthy that overexpressed RIP3 can induce caspase activation and apoptotic cell death (Sun et al., 1999; Yu et al., 1999).

RIP3 is required for necroptotic cell death, which is distinct from other forms of necrosis that occur independent of RIP3. Necrotic cells release ATP that can bind to the purinergic P2X7 receptor of healthy cells and can cause NLRP3-caspase-1 inflammasome activation (Ghiringhelli et al., 2009; Iyer et al., 2009; McDonald et al., 2010). In some situations, necrosis induction or lysosomal destabilization may also activate NLRP3-caspase-1 through an ill-defined pathway that is blocked by the cathepsin B inhibitor CA-074-Me (Halle et al., 2008; Hornung et al., 2008; Li et al., 2009). However, we found no evidence implicating lysosomal function or cathepsin activity in Smac mimetic-induced IL-1 activation. Our data also show that Smac mimetic-induced IL-1 activation occurs prior to any disruption of plasma membrane integrity and is not affected by the deletion of P2X7. Therefore, we conclude that RIP3 signaling, but not cell death, is sufficient for IL-1β maturation and secretion.

EXPERIMENTAL PROCEDURES

Macrophage and Dendritic Cell Preparation, Stimulation, and IL-1 Analysis

Bone marrow myeloid progenitor cells were differentiated into BMDMs for 7 days in DME (Invitrogen) (37°C, 5% CO_2) supplemented with 10% FBS and 20% L929 conditioned medium or BMDCs for 8 days in DME supplemented with 10% FBS and 20 ng/ml of GM-CSF (Immunotools). After differentiation, adherent (BMDMs) or nonadherent (BMDCs) cells were replated at 2 × 10^5 cells/well (96-well plates) or 5–6 × 10^5 cells/well (24-well plates) and primed with 20 ng/ml of ultrapure LPS for 3 hr. Cells were stimulated as indicated and cell supernatant collected for ELISA (IL-1α, IL-1β, or TNF; R&D systems) and immunoblot analysis. Cells lysates and supernatants were separated on 8%, 12%, or gradient 4%–12% polyacrylamide gels (Invitrogen) and protein transferred to nitrocellulose (Amersham) membranes. Membranes were blocked with 5% skim milk in PBST (PBS containing 0.05% Tween 20) and all primary antibody incubations were performed overnight and secondary antibody incubations were performed for 1–2 hr. Membranes were washed 5–8 times in PBST after all antibody incubations. Antibody dilutions were performed with 5% skim milk in PBST. Mice were treated in accordance with the Swiss Federal Veterinary Office guidelines (Switzerland) and under conditions approved by the Walter and Eliza Hall Institute Animal Ethics Committee (Australia).

Reactive Oxygen Species Measurement

Bone marrow-derived macrophages were seeded at 5 × 10^5 cells/well (24-well) tissue culture plates. Cells were labeled with 5 μM of CellROX Deep Red (Invitrogen) for 30 min (37°C, 5% CO_2), washed twice, and then stimulated as indicated in the figure legends. Cells were then harvested and analyzed by flow cytometry. Viable cells (PI negative) were quantified for ROS production by calculating the mean fluorescence intensity (MFI) of 5,000 events with Weasel V2.0 analysis software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.immuni.2012.01.012.

ACKNOWLEDGMENTS

Thanks to M. McKinlay and S.K. Chunduru for Smac mimetic compound (TetraLogic Pharmaceuticals) and D. Vaux, M. Leverkus, K. Schroder, and B. Croker for advice. We would also like to thank D. Huang (Walter and Eliza Hall Institute, Australia) for providing Bax and Bak knockout mice, V. Dixit (Genentech, USA) for *Ripk3*$^{-/-}$ mice, A. Strasser (Walter and Eliza Hall Institute, Australia) for *Bid*$^{-/-}$ mice, H. Korner (James Cook University, Australia) for *Tnf*$^{-/-}$ and *Tnfrsf1a*$^{-/-}$ mice, B. Crocker (Walter and Eliza Hall Institute, Australia) for the pEF IL-1-FLAG cDNA, and E. Latz (University of Massachusetts Medical School, USA) for the v-myc/v-raf immortalized macrophages. J.E.V. was supported by Human Frontiers and NHMRC C. J. Martin fellowships. G.H. is supported by a Deutsche Krebshilfe/Mildred Scheel-Stiftung grant and I.G. is supported by an EMBO Long Term Fellowship. J.S. is on the scientific advisory board at TetraLogic pharmaceuticals and is supported by NHMRC grants 541901, 541902, and 602516. L.O. is supported by NHMRC grant 1009145. This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS.

Received: October 11, 2011
Revised: December 21, 2011
Accepted: January 12, 2012
Published online: February 23, 2012

REFERENCES

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A., et al. (2009). Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J. Immunol. *183*, 787–791.

Bauernfeind, F., Bartok, E., Rieger, A., Franchi, L., Núñez, G., and Hornung, V. (2011). Cutting edge: reactive oxygen species inhibitors block priming, but not activation, of the NLRP3 inflammasome. J. Immunol. *187*, 613–617.

Bertrand, M.J., Doiron, K., Labbé, K., Korneluk, R.G., Barker, P.A., and Saleh, M. (2009). Cellular inhibitors of apoptosis cIAP1 and cIAP2 are required for innate immunity signaling by the pattern recognition receptors NOD1 and NOD2. Immunity *30*, 789–801.

Cho, Y.S., Challa, S., Moquin, D., Genga, R., Ray, T.D., Guildford, M., and Chan, F.K. (2009). Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. Cell *137*, 1112–1123.

Darding, M., Feltham, R., Tenev, T., Bianchi, K., Benetatos, C., Silke, J., and Meier, P. (2011). Molecular determinants of Smac mimetic induced degradation of cIAP1 and cIAP2. Cell Death Differ. *18*, 1376–1386.

Degterev, A., Hitomi, J., Germscheid, M., Ch'en, I.L., Korkina, O., Teng, X., Abbott, D., Cuny, G.D., Yuan, C., Wagner, G., et al. (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. *4*, 313–321.

Dynek, J.N., and Vucic, D. (2010). Antagonists of IAP proteins as cancer therapeutics. Cancer Lett., in press, Published online August 2, 2010. 10.1016/j.canlet.2010.06.013.

Eckelman, B.P., and Salvesen, G.S. (2006). The human anti-apoptotic proteins cIAP1 and cIAP2 bind but do not inhibit caspases. J. Biol. Chem. *281*, 3254–3260.

Eder, C. (2009). Mechanisms of interleukin-1beta release. Immunobiology *214*, 543–553.

Eisenbarth, S.C., Colegio, O.R., O'Connor, W., Sutterwala, F.S., and Flavell, R.A. (2008). Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature *453*, 1122–1126.

Feltham, R., Bettjeman, B., Budhidarmo, R., Mace, P.D., Shirley, S., Condon, S.M., Chunduru, S.K., McKinlay, M.A., Vaux, D.L., Silke, J., and Day, C.L. (2011). Smac mimetics activate the E3 ligase activity of cIAP1 protein by promoting RING domain dimerization. J. Biol. Chem. *286*, 17015–17028.

Feng, S., Yang, Y., Mei, Y., Ma, L., Zhu, D.E., Hoti, N., Castanares, M., and Wu, M. (2007). Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. Cell. Signal. *19*, 2056–2067.

Feoktistova, M., Geserick, P., Kellert, B., Dimitrova, D.P., Langlais, C., Hupe, M., Cain, K., MacFarlane, M., Häcker, G., and Leverkus, M. (2011). cIAPs block ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms. Mol. Cell *43*, 449–463.

Gardam, S., Turner, V.M., Anderton, H., Limaye, S., Basten, A., Koentgen, F., Vaux, D.L., Silke, J., and Brink, R. (2011). Deletion of cIAP1 and cIAP2 in murine B lymphocytes constitutively activates cell survival pathways and inactivates the germinal center response. Blood *117*, 4041–4051.

Ghiringhelli, F., Apetoh, L., Tesniere, A., Aymeric, L., Ma, Y., Ortiz, C., Vermaelen, K., Panaretakis, T., Mignot, G., Ullrich, E., et al. (2009). Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nat. Med. *15*, 1170–1178.

Gloire, G., Legrand-Poels, S., and Piette, J. (2006). NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem. Pharmacol. *72*, 1493–1505.

Grech, A.P., Amesbury, M., Chan, T., Gardam, S., Basten, A., and Brink, R. (2004). TRAF2 differentially regulates the canonical and noncanonical pathways of NF-kappaB activation in mature B cells. Immunity *21*, 629–642.

Gyrd-Hansen, M., and Meier, P. (2010). IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. Nat. Rev. Cancer *10*, 561–574.

Halle, A., Hornung, V., Petzold, G.C., Stewart, C.R., Monks, B.G., Reinheckel, T., Fitzgerald, K.A., Latz, E., Moore, K.J., and Golenbock, D.T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. Nat. Immunol. *9*, 857–865.

He, S., Wang, L., Miao, L., Wang, T., Du, F., Zhao, L., and Wang, X. (2009). Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell *137*, 1100–1111.

He, S., Liang, Y., Shao, F., and Wang, X. (2011). Toll-like receptors activate programmed necrosis in macrophages through a receptor-interacting kinase-3-mediated pathway. Proc. Natl. Acad. Sci. USA *108*, 20054–20059.

Hoffman, H.M., and Brydges, S.D. (2011). Genetic and molecular basis of inflammasome-mediated disease. J. Biol. Chem. *286*, 10889–10896.

Hornung, V., and Latz, E. (2010). Critical functions of priming and lysosomal damage for NLRP3 activation. Eur. J. Immunol. *40*, 620–623.

Hornung, V., Bauernfeind, F., Halle, A., Samstad, E.O., Kono, H., Rock, K.L., Fitzgerald, K.A., and Latz, E. (2008). Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. Nat. Immunol. *9*, 847–856.

Iyer, S.S., Pulskens, W.P., Sadler, J.J., Butter, L.M., Teske, G.J., Ulland, T.K., Eisenbarth, S.C., Florquin, S., Flavell, R.A., Leemans, J.C., and Sutterwala, F.S. (2009). Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome. Proc. Natl. Acad. Sci. USA *106*, 20388–20393.

Jin, C., and Flavell, R.A. (2010). Molecular mechanism of NLRP3 inflammasome activation. J. Clin. Immunol. *30*, 628–631.

Jin, Z., Li, Y., Pitti, R., Lawrence, D., Pham, V.C., Lill, J.R., and Ashkenazi, A. (2009). Cullin3-based polyubiquitination and p62-dependent aggregation of caspase-8 mediate extrinsic apoptosis signaling. Cell *137*, 721–735.

Kaiser, W.J., Upton, J.W., Long, A.B., Livingston-Rosanoff, D., Daley-Bauer, L.P., Hakem, R., Caspary, T., and Mocarski, E.S. (2011). RIP3 mediates the embryonic lethality of caspase-8-deficient mice. Nature *471*, 368–372.

Kang, T.B., Ben-Moshe, T., Varfolomeev, E.E., Pewzner-Jung, Y., Yogev, N., Jurewicz, A., Waisman, A., Brenner, O., Haffner, R., Gustafsson, E., et al. (2004). Caspase-8 serves both apoptotic and nonapoptotic roles. J. Immunol. *173*, 2976–2984.

Kayagaki, N., Warming, S., Lamkanfi, M., Vande Walle, L., Louie, S., Dong, J., Newton, K., Qu, Y., Liu, J., Heldens, S., et al. (2011). Non-canonical inflammasome activation targets caspase-11. Nature *479*, 117–121.

Lamkanfi, M., Mueller, J.L., Vitari, A.C., Misaghi, S., Fedorova, A., Deshayes, K., Lee, W.P., Hoffman, H.M., and Dixit, V.M. (2009). Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. J. Cell Biol. *187*, 61–70.

Li, H., Ambade, A., and Re, F. (2009). Cutting edge: Necrosis activates the NLRP3 inflammasome. J. Immunol. *183*, 1528–1532.

Lin, Y., Devin, A., Rodriguez, Y., and Liu, Z.G. (1999). Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. Genes Dev. *13*, 2514–2526.

Lüthi, A.U., Cullen, S.P., McNeela, E.A., Duriez, P.J., Afonina, I.S., Sheridan, C., Brumatti, G., Taylor, R.C., Kersse, K., Vandenabeele, P., et al. (2009). Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. Immunity *31*, 84–98.

Maelfait, J., Vercammen, E., Janssens, S., Schotte, P., Haegman, M., Magez, S., and Beyaert, R. (2008). Stimulation of Toll-like receptor 3 and 4 induces interleukin-1beta maturation by caspase-8. J. Exp. Med. *205*, 1967–1973.

Mao, A.P., Li, S., Zhong, B., Li, Y., Yan, J., Li, Q., Teng, C., and Shu, H.B. (2010). Virus-triggered ubiquitination of TRAF3/6 by cIAP1/2 is essential for induction of interferon-beta (IFN-beta) and cellular antiviral response. J. Biol. Chem. *285*, 9470–9476.

McDonald, B., Pittman, K., Menezes, G.B., Hirota, S.A., Slaba, I., Waterhouse, C.C., Beck, P.L., Muruve, D.A., and Kubes, P. (2010). Intravascular danger signals guide neutrophils to sites of sterile inflammation. Science *330*, 362–366.

Menu, P., and Vince, J.E. (2011). The NLRP3 inflammasome in health and disease: the good, the bad and the ugly. Clin. Exp. Immunol. *166*, 1–15.

Miwa, K., Asano, M., Horai, R., Iwakura, Y., Nagata, S., and Suda, T. (1998). Caspase 1-independent IL-1beta release and inflammation induced by the apoptosis inducer Fas ligand. Nat. Med. *4*, 1287–1292.

Nakahira, K., Haspel, J.A., Rathinam, V.A., Lee, S.J., Dolinay, T., Lam, H.C., Englert, J.A., Rabinovitch, M., Cernadas, M., Kim, H.P., et al. (2011). Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. Nat. Immunol. *12*, 222–230.

O'Donnell, M.A., Perez-Jimenez, E., Oberst, A., Ng, A., Massoumi, R., Xavier, R., Green, D.R., and Ting, A.T. (2011). Caspase 8 inhibits programmed necrosis by processing CYLD. Nat. Cell Biol. *13*, 1437–1442.

Oberst, A., Dillon, C.P., Weinlich, R., McCormick, L.L., Fitzgerald, P., Pop, C., Hakem, R., Salvesen, G.S., and Green, D.R. (2011). Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis. Nature *471*, 363–367.

Pelegrin, P., Barroso-Gutierrez, C., and Surprenant, A. (2008). P2X7 receptor differentially couples to distinct release pathways for IL-1beta in mouse macrophage. J. Immunol. *180*, 7147–7157.

Rock, K.L., Latz, E., Ontiveros, F., and Kono, H. (2010). The sterile inflammatory response. Annu. Rev. Immunol. *28*, 321–342.

Schroder, K., and Tschopp, J. (2010). The inflammasomes. Cell *140*, 821–832.

Sun, X., Lee, J., Navas, T., Baldwin, D.T., Stewart, T.A., and Dixit, V.M. (1999). RIP3, a novel apoptosis-inducing kinase. J. Biol. Chem. *274*, 16871–16875.

Tenev, T., Bianchi, K., Darding, M., Broemer, M., Langlais, C., Wallberg, F., Zachariou, A., Lopez, J., MacFarlane, M., Cain, K., and Meier, P. (2011). The ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs. Mol. Cell *43*, 432–448.

Tseng, P.H., Matsuzawa, A., Zhang, W., Mino, T., Vignali, D.A., and Karin, M. (2010). Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. Nat. Immunol. *11*, 70–75.

Upton, J.W., Kaiser, W.J., and Mocarski, E.S. (2010). Virus inhibition of RIP3-dependent necrosis. Cell Host Microbe *7*, 302–313.

van de Veerdonk, F.L., Smeekens, S.P., Joosten, L.A., Kullberg, B.J., Dinarello, C.A., van der Meer, J.W., and Netea, M.G. (2010). Reactive oxygen species-independent activation of the IL-1beta inflammasome in cells from patients with chronic granulomatous disease. Proc. Natl. Acad. Sci. USA *107*, 3030–3033.

Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., and Kroemer, G. (2010). Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat. Rev. Mol. Cell Biol. *11*, 700–714.

Vanlangenakker, N., Vanden Berghe, T., Bogaert, P., Laukens, B., Zobel, K., Deshayes, K., Vucic, D., Fulda, S., Vandenabeele, P., and Bertrand, M.J. (2011). cIAP1 and TAK1 protect cells from TNF-induced necrosis by

preventing RIP1/RIP3-dependent reactive oxygen species production. Cell Death Differ. *18*, 656–665.

Varfolomeev, E.E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J.S., Mett, I.L., Rebrikov, D., Brodianski, V.M., Kemper, O.C., Kollet, O., et al. (1998). Targeted disruption of the mouse caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity *9*, 267–276.

Varfolomeev, E., Blankenship, J.W., Wayson, S.M., Fedorova, A.V., Kayagaki, N., Garg, P., Zobel, K., Dynek, J.N., Elliott, L.O., Wallweber, H.J., et al. (2007). IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell *131*, 669–681.

Vince, J.E., Wong, W.W., Khan, N., Feltham, R., Chau, D., Ahmed, A.U., Benetatos, C.A., Chunduru, S.K., Condon, S.M., McKinlay, M., et al. (2007). IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell *131*, 682–693.

Vince, J.E., Chau, D., Callus, B., Wong, W.W., Hawkins, C.J., Schneider, P., McKinlay, M., Benetatos, C.A., Condon, S.M., Chunduru, S.K., et al. (2008). TWEAK-FN14 signaling induces lysosomal degradation of a cIAP1-TRAF2 complex to sensitize tumor cells to TNFalpha. J. Cell Biol. *182*, 171–184.

Vince, J.E., Pantaki, D., Feltham, R., Mace, P.D., Cordier, S.M., Schmukle, A.C., Davidson, A.J., Callus, B.A., Wong, W.W., Gentle, I.E., et al. (2009). TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf-kappab and to prevent tnf-induced apoptosis. J. Biol. Chem. *284*, 35906–35915.

Wallach, D., Kovalenko, A., and Kang, T.B. (2011). 'Necrosome'-induced inflammation: must cells die for it? Trends Immunol. *32*, 505–509.

Weber, A., Kirejczyk, Z., Besch, R., Potthoff, S., Leverkus, M., and Häcker, G. (2010). Proapoptotic signalling through Toll-like receptor-3 involves TRIF-dependent activation of caspase-8 and is under the control of inhibitor of apoptosis proteins in melanoma cells. Cell Death Differ. *17*, 942–951.

Yu, P.W., Huang, B.C., Shen, M., Quast, J., Chan, E., Xu, X., Nolan, G.P., Payan, D.G., and Luo, Y. (1999). Identification of RIP3, a RIP-like kinase that activates apoptosis and NFkappaB. Curr. Biol. *9*, 539–542.

Zhang, D.W., Shao, J., Lin, J., Zhang, N., Lu, B.J., Lin, S.C., Dong, M.Q., and Han, J. (2009). RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science *325*, 332–336.

Zhou, R., Yazdi, A.S., Menu, P., and Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. Nature *469*, 221–225.